Nestec Ltd.
Technical Assistance

Nestlé

Laboratory Instructions

Effective date:

Date issued:
1996-10-16

Title:

MILK POWDERS DETERMINATION OF PROTEINS AND PROTEIN FRAGMENTS BY ELECTROPHORESIS (SDS-PAGE WITH PHASTSYSTEM AND SILVER STAINING)

LI number
08.086 (2 08086)

References:
R&D/QS
CHi/CRa/cva

Pages: 15

Keywords: SDS-PAGE, hypoallergenic milks, protein hydrolysates

1 SCOPE AND FIELD OF APPLICATION

Description of a method for the determination of undigested and partially digested proteins (MW>14kD) in protein hydrolysates, products containing protein hydrolysates such as hypoallergenic milks, extensively hydrolysed formulas, and starch for hypoallergenic formulas.

Compared to LI-08.082, this method is much more sensitive and allows to detect also traces of proteins and peptides in extensively hydrolysed formulas such as Alfaré.

2 PRINCIPLE OF THE METHOD

Protein denaturation with sodium dodecyl sulphate (SDS), reduction of sulfur-sulfur bonds with dithiotreitol (DTT) and alkylation of SH-groups with iodoacetamide. Separation of proteins and protein fragments by SDS-PAGE (sodium dodecyl sulphate - polyacrylamide gel electrophoresis) according to Laemmli (1970): when performing this type of electrophoresis the gel consists of a stacking and a separation gel. The buffer composition, the pore size and the pH of the stacking gel result in a concentration of the deposited proteins (isotachophoretic effect) and the diffusion of the bands into the separation gel is considerably reduced. Separation of the proteins according to their molecular weight. Silver staining of the proteins and evaluation by visual comparison with molecular weight markers and a reference sample.

3 REAGENTS AND MATERIALS

Commercial references are only a guideline. Numbers in the margin refer either to the laboratory material catalogue or to that of Merck's chemicals and reagents.

3.1 Reagents

Before using chemicals refer to the Sigma/Aldrich Guide to Chemical Safety and/or other adequate manuals or safety data sheets approved by your local authorities. Read carefully the safety instructions given in Enclosure 1. More detailed safety information can also be obtained from R&D/QS.

Electrophoresis Calibration Kit Low Molecular Weight Protein Standard	Pharmacia nr. 17-0446-01
Phastgel separation medium Homogeneous 20, MW range 2-150 KD	Pharmacia nr. 17-0624-01
Phastgel SDS Buffer strips	Pharmacia nr. 17-0516-01
Phastgel Sample Applicator 8/1	Pharmacia nr. 18-1618-01

63 - Acetic acid glacial 100 %
317 - Ethanol p.a., Fluka nr. 02860
317 - Hydrochloric acid fuming 37 %
1512 - Silver nitrate
4001 - Formaldehyde solution min. 35 %
4094 - Glycerol (about 87 %) GR
6009 - Methanol p.a.
6267 - Sodium acetate trihydrate
6392 - Sodium carbonate anhydrous
6516 - Sodium thiosulfate pentahydrate
8122 - Bromophenol blue
8382 - Tris(hydroxymethyl)aminomethane GR
Sodium dodecyl sulphate salt, Serva nr. 20760
Dithiothreitol, Pharmacia nr. 80-1128-79
Iodoacetamide, Fluka nr. 57 670
820603 - Glutaraldehyde 25 % solution

3.2 Materials

1336 - Flasks with screw neck "Iso", with pierced stopper, 1 l
8076 Forceps for microscope slides
Eppendorf Save-Lock reaction tubes, 2 ml, Eppendorf nr. 0030 120.094
Microtubes Eppendorf, 1,5 ml, Eppendorf nr. 0030.102.002
Fast Electrophoresis System PHASTSYSTEM, Pharmacia nr. 18-1018-24
Eppendorf Thermomixer, Eppendorf nr. 5437 000.010
93301 - Magnetic stirrer, heated, IKA RET
116307 - Erlenmeyer flask, RN, 100 ml
116315 - idem, 1 000 ml
304102 - Volumetric flasks, class A, RN stopper, PE, 20 ml
304105 - idem, 100 ml
304106 - idem, 200 ml
304109 - idem, 1 000 ml
304110 - idem, 2 000 ml
334802 - Micropipette Socorex, adjustable, 50 - 200 µl
334803 - idem, 200 - 1000 µl
334804 - PP tips, yellow, 5 - 200 µl
334805 - PP tips blue, 200 - 1 000 µl
334901 - idem, 0,5 - 5 ml

Varipipette 4810, adjustable, 1 - 10 µl, Eppendorf nr. 4810.000.045
334902 - PP tips white, 0,5 - 5 ml
Eppendorf tips, 10 µl, Eppendorf nr. 0030.063.651

4 PREPARATION OF REAGENTS

4.1 Incubation buffer

4.1.1 Tris buffer, pH 6,80 (1,0 M)

Into a 100 ml beaker weigh 12,1 g Tris(hydroxymethyl)aminomethane. Dissolve in about 80 ml water. Adjust the pH with 6M hydrochloric acid (pH-meter!). As the pH approaches near 7,0, adjust carefully drop by drop to exactly 6,80. Transfer the solution to a 100 ml volumetric flask and complete to the mark with water.
Stored at 4°C, this solution is stable for 1 month.

4.1.2 SDS solution, 20 g/100 ml

Weigh 20 g SDS into a 100 ml volumetric flask. Dissolve in water and complete to the mark.
Stored at room temperature, this solution is stable for 3 months.

4.1.3 Bromophenol blue solution, 0,05 g/100 ml

Weigh 50 mg bromophenol blue into a 100 ml volumetric flask. Dissolve in water and complete to the mark.
Stored at room temperature, this solution is stable for 3 months.

4.1.4 Incubation buffer (stock solution)

Into a 100 ml Erlenmeyer flask with ground neck, pipette in the following order:

- 5 ml Tris buffer (4.1.1)
- 12,5 ml SDS solution (4.1.2)
- 10 ml bromophenol blue solution (4.1.3)
- 5,6 ml glycerol (87%)
  14,4 ml water

Mix well. Stored at room temperature, this solution is stable for one week.

4.1.5 Incubation buffer (concentrated working solution)

Just before use, pipette into a 10 ml beaker 2 ml of buffer 4.1.4 and add 75 mg DTT. Dissolve completely by mixing. Prepare the volume dependent on the number of samples to be incubated (37,5 mg DTT/ml buffer 4.1.4).

4.1.6 Incubation buffer (diluted working solution)

Just before use dilute 1 volume of solution 4.1.5 with 4 volumes of water.

4.1.7 Sample dilution buffer

Dilute 1 volume of solution 4.1.4 with 4 volumes of water.
Stored at room temperature, this solution is stable for one week.

4.1.8 Hydrochloric acid solution, about 6M

Into a suitable flask containing 1 volume of water, slowly pour 1 volume of fuming HCl (37 %). Mix and allow to cool.

4.2 Alkylation solution (2,0 M)

4.2.1 Tris buffer, pH 8,0

Into a 100 ml beaker weigh 12,1 g Tris(hydroxymethyl)aminomethane. Dissolve in about 25-30 ml water. Adjust the pH with 6 M HCl (pH-meter!). As pH approaches 8,2 adjust carefully drop by drop to exactly 8,0. Transfer the solution to a 50 ml volumetric flask and complete to the mark with water.
Stored at 4 °C, this solution is stable for 1 month.

4.2.2 Alkylation solution

Just before use, weigh 540 mg iodoacetamide into a test tube and dissolve in 3 ml Tris buffer (4.2.1) by mixing on a Vortex.

4.3 Solutions for silver staining

Note : It is recommended to prepare solutions 4.3.5 (Fixing sol.), 4.3.6 (Staining sol.) and 4.3.7 (Developing sol.) at least 48 hours before use in order to obtain an optimal activity and sensitivity of staining.

Furthermore, to avoid contamination of these solutions, it is advisable to treat all the glass flasks with detergent (e.g. RBS) and to rinse then well with distilled water before use.

When all the solutions are ready to use (also washing solutions), transfer them into one-litre flasks with pierced stoppers, also treated with detergent and rinsed, and directly connect them to the to the PHASTSYSTEM development Unit.

4.3.1 Wash solution 1 (30 % ethanol and 10 % acetic acid)

Into a 1 000 ml volumetric flask, pipette 300 ml ethanol and 100 ml glacial acetic acid. Complete to the mark with water.
Stored at room temperature, this solution is stable for 1 month.

4.3.2 Wash solution 2

Nano-pure water : Use either redistilled water or purified water by a Milli-Q system from Millipore (or equivalent), of a resistivity ≥15 MΩ$cm.

4.3.3 Wash solution 3 (5 % acetic acid)

Into a 1 000 ml volumetric flask, pipette 50 ml glacial acetic acid. Complete to the mark with water.
Stored at room temperature, this solution is stable for 1 month.

4.3.4 Preserving solution (~ 26 % glycerol)

Into a 1 000 ml volumetric flask, pipette 300 ml glycerol about 87 %. Complete to the mark with water.
Stored at room temperature, this solution is stable for 1 month.

4.3.5 Fixing solution

Stock solution : Into a 1 000 ml volumetric flask, pipette 300 ml ethanol, 100 ml of a 4M sodium acetate solution (54,43 g sodium acetate trihydrate/100 ml water) and 500 ml water. Adjust to pH 6,0 with acetic acid (pH-meter !), then complete to the mark with water.

Final solution : Into a 1 000 ml volumetric flask, weigh 1,6 g sodium thiosulfate pentahydrate and pipette 20 ml glutaraldehyde 25 % solution. Dissolve and complete to the mark with stock solution.

Stored at room temperature, the final solution is stable for 1 month.

4.3.6 Staining solution

Stock solution : Into a 1 000 ml volumetric flask, weigh 1,00 g silver nitrate. Dissolve and complete to the mark with water.

Final solution : Add 265 µl of formaldehyde sol. min. 35 % to 1 000 ml stock solution.

Stored at room temperature, the final solution is stable for 1 month.

4.3.7 Developing solution

Stock solution : Into a 1 000 ml volumetric flask, weigh 25,00 g anhydrous sodium carbonate. Dissolve and complete to the mark with water.

Final solution : Add 424 µl of formaldehyde sol. min. 35 % to 1 000 ml stock solution.

Stored at room temperature, the final solution is stable for 1 month.

4.4 Protein reference solution

Dissolve the contents of a "Protein Calibration Kit" vial in 1 000 µl incubation buffer 4.1.6. Transfer the whole volume into a reaction tube, close the tube and incubate the solution for 10 min. in a boiling water bath or for 15 min. in a Thermomixer set at 95 °C. Cool to room temperature and add 200 µl alkylation solution (4.2.2). Allow to react for 20 min. in the dark. Divide this solution into 50 µl portions, by pipetting 50 µl in Save-Lock reaction tubes.

Stored at -20 °C, the solution is stable for 1 month.

It contains the following amounts of proteins per 1 200 µl solution :

121 µg α-lactalbumin, molecular weight	MW = 14 kD
80 µg soy trypsin inhibitor,	MW = 20,1 kD
83 µg carbonic anhydrase,	MW = 30 kD
147 µg ovalbumin	MW = 43 kD
83 µg BSA (bovine serum albumin)	MW = 67 kD
64 µg phosphorylase b	MW = 94 kD

5 PROCEDURE

With each series of analysis run an appropriate reference sample.

5.1 Preparation of the test portion

5.1.1 Powdered products

Into a test tube with screw cap or into a Save-Lock reaction tube, weigh a test portion corresponding to 1,2 mg N. For a product containing 1,96% total nitrogen, weigh 61,2 mg.

5.1.2 Starch for hypoallergenic milk

Into a test tube with screw cap or into a Save-Lock reaction tube, weigh a test portion that corresponds, after having diluted the final extract 15 times, to 0,067 µg N of finished product (i.e.

hypoallergenic milk with starch) deposited on the gel. For a finished product containing 2,38 % total nitrogen and 14% starch, weigh 14,1 mg starch.

5.1.3 Liquid products and protein hydrolysates (liquid and powdered, e.g. LAD)

Into a 100 ml volumetric flask weigh a test portion corresponding to 0,15 g N. For a product containing 0,26 % total nitrogen, weigh 58,0 g.
Fill up to the mark with water and mix well.

5.2 Incubation

5.2.1 Powdered products and starch

Using a pipette add 1 ml incubation buffer (diluted working solution 4.1.6) to the test portion (5.1.1 and 5.1.2). Close the tube and mix well on a Vortex. Incubate the solution either for 10 min. in a boiling water bath or for 15 min. in a Thermomixer set at 95 °C.

5.2.2 Liquid products and protein hydrolysates

Into a test tube with screw cap or into a Save-Lock reaction tube pipette 320 µl solution (5.1.3) and add, by means of a pipette 80 µl incubation buffer (concentrated working solution, 4.1.5). Close the tube and mix well on a Vortex. Incubate the solution either for 10 min. in a boiling water bath or for 15 min. in a Thermomixer set at 95 °C.

5.3 Alkylation

Allow the tubes to cool for some minutes and pipette 200 µl alkylation solution (4.2.2) into each tube incubated according to 5.2.1, and 80 µl alkylation solution (4.2.2) into each tube incubated according to 5.2.2. Mix. Close again and allow to react for 20 min. in the dark.

At this step, if the electrophoresis cannot be performed on the day of preparation, the samples can be stored overnight at +4 °C or for 1 month at -20 °C.

5.4 Working dilutions of protein reference solutions and sample solutions

5.4.1 Protein reference solutions

a) Dilution to obtain about 0,8 ng of each marker protein / µl :
Bring one vial containing 50 µl of solution 4.4 to room temperature. Using a pipette add 4,95 ml of solution 4.1.7 and mix well.

b) Dilution to obtain about 0,08 ng of each marker protein / µl :
Mix 1 volume of solution a) with 9 volumes of solution 4.1.7

Prepare these solutions on the day of use.

5.4.2 Sample solutions

5.4.2.1 Starch

a) Dilution to obtain a solution containing the amount of starch present in 0,067 µg N of finished product in 1 µl :
To 50 µl of solution obtained under 5.3 add, using a pipette, 700 µl of solution 4.1.7

b) Dilution to obtain a solution containing the amount of starch present in 0,033 µg N of finished product in 1 µl :
Mix 1 volume of solution a) with 1 volume of solution 4.1.7

5.4.2.2 Powdered and liquid products - hypoallergenic formulas, protein hydrolysates (H.A.)

a) Dilution to obtain a solution containing 0,067 µg N in 1 µl:
To 50 µl of solution obtained under 5.3 add, using a pipette, 700 µl of solution 4.1.7

b) Dilution to obtain a solution containing 0,033 µg N in 1 µl:
Mix 1 volume of solution a) with 1 volume of solution 4.1.7

5.4.2.3 Powdered and liquid products - extensively hydrolysed formulas (e.g. Alfaré), protein hydrolysates (e.g. LAD)

a) Solution containing 1 µg N in 1 µl : Use solution obtained under 5.3 as is ready for use.

b) Dilution to obtain a solution containing 0,5 µg N in 1 µl :
Mix 1 volume of solution obtained under 5.3 (a) with 1 volume of solution 4.1.7

5.5 Electrophoresis with PHASTSYSTEM

5.5.1 Electrophoretic separation

Wear gloves to avoid contact with the gels and to prevent contaminations. It is advisable to use non powdered gloves.

For particular details of use concerning the PHASTSYSTEM, refer to the instruction manual (Pharmacia).

Bring one or two gels (depending on the number of samples) and the appropriate number of SDS-buffer strips (two for one gel and four for two gels) to room temperature some minutes before beginning electrophoresis.

Also bring samples to room temperature if stored at -20 °C or +4 °C.

Switch on the PHASTSYSTEM and set the temperature of the separation unit to 15 °C.

Remove the gels from their pocket and bend up the small tab in order to hold them easily with a flat tweezer.

Carefully apply the gels in the separation unit on the gel bed area and do not forget to remove the protecting film.

Position the buffer strips holder over the gels and place the SDS-buffer strips in the holder.

Pull down the electrode holder over the SDS-buffer strips and press gently to obtain an optimal contact between the electrodes and the buffer strips to the gels.

Pull down the sample applicator arm and close the separation compartment lid.

Keep the separation compartment closed until samples are ready for electrophoresis, in order to prevent contaminations on the gels (contact with dust in the air,...).

Prepare the sample-well stamp onto a table with the wells facing upwards.

Cover with a piece of parafilm and run a pen or other hard object along the lane of eight wells, in order to make depressions in the parafilm.

Pipette 3 µl of each sample and protein reference solutions directly into the corresponding wells on the parafilm. Make sure there are no air bubbles.

Lower a sample applicator 8/1 (8 samples / 1 µl of each on the gel) to the surface of the samples, so that, just by touching, they climb into the applicator capillaries. Avoid samples climbing the sides of the applicator.

This operation should be carried out with a steady hand on a very stable surface.

The amount of samples loaded on the applicator, when applied to the gel, corresponds to approximately 1 µl of each sample.

Slide the sample applicator into the applicator arm, into the slots nearest the cathode (-).

Do not press down on the applicator arm otherwise the samples may touch the gel surface.

Close the separation compartment lid and start the following separation program :

SEPARATION PROGRAM :

SAMPLE APPL.	DOWN AT	X.2	000 Vh	
SAMPLE APPL.	UP AT	X.3	000 Vh	
EXTRA ALARM TO SOUND AT		X.4	000 Vh	

SEP. X.1	250 V	05.0 mA	3 W	15°C	001 Vh
SEP. X.2	250 V	03.0 mA	3 W	15°C	002 Vh
SEP. X.3	250 V	10.0 mA	3 W	15°C	116 Vh
SEP. X.4	050 V	00.5 mA	2 W	15°C	000 Vh

The total run time is about 35 minutes.

5.5.2 Development

Before using the development unit, make sure the chamber and tubings are clean, to avoid precipitation on the gels.
It is advisable to rinse the system just before developing the gels (for example during the separation), once with distilled water and with each solution for silver staining.

At the end of the electrophoretic separation, open the separation compartment lid, discard the sample applicators and the SDS-buffer strips and remove the gels from the gel bed, holding them with a flat pincer.
Place the gels face to face into the development chamber.

Close the development unit and start the following development program :

DEVELOPMENT PROGRAM :

	SOLUTION	IN	OUT	t(min)	T(°C)
Step 1	WASH SOLUTION 1	3	0	4	50
Step 2	WASH SOLUTION 1	3	0	4	50
Step 3	FIXING SOLUTION	4	0	8	40
Step 4	WASH SOLUTION 2	5	0	3	50
Step 5	WASH SOLUTION 2	5	0	3	50
Step 6	WASH SOLUTION 2	5	0	3	50
Step 7	WASH SOLUTION 2	5	0	4	50
Step 8	WASH SOLUTION 2	5	0	4	50
Step 9	STAINING SOLUTION	6	0	10	40
Step 10	DEVELOPING SOLUTION	7	0	0.4	30
Step 11	DEVELOPING SOLUTION	7	0	9	30
Step 12	WASH SOLUTION 3	8	0	2	50
Step 13	WASH SOLUTION 2	5	0	3	50
Step 14	PRESERVING SOLUTION	9	0	3	50
Step 15	WASH SOLUTION 2	5	0	0.1	40

Note : Depending on the difference of sensitivity from an assay to another, incubation time can be modified for Step 11 (Developing step) between 6' to 11'.
Usually, sensitivity increases when prolonging incubation time, and vice-versa.

5.5.3 Drying the gels

After development, remove the gels from the development chamber and allow them to dry overnight at room temperature. When dried, the gels can be kept indefinitely.

6 CALCULATION, EXPRESSION AND INTERPRETATION OF THE RESULTS

6.1 Visual evaluation of the gel and expression of results

Staining sensitivity and evaluation of samples is reliable only if at least all but one bands of the protein reference solution containing about 0,08 ng of each marker protein/µl (5.4.1) are visible.

Inspect the electropherograms of both sample concentrations (0,033 and 0,067 µg N or 0,5 and 1 µg N respectively).

Observe whether there are any distinct or diffuse bands corresponding to a MW > 14 kD in both samples and reference sample.

Observe whether there are any distinct bands corresponding to the molecular weights of α-lactalbumin, ß-lactoglobulin (MW, ≈ 18 kD), BSA or trypsin (MW ≈ 24 kD).

In the case of a positive result, note the proteins observed or the approximate molecular weights of the bands, their intensity (weak, strong) and their appearance (distinct, diffuse).

Note whether the intensity of the bands observed in the sample is stronger than in the reference sample.

6.2 Autocontrol procedure

Reference sample:

Include in each series of analysis a reference sample with a known electrophoretic profile.
Stored at -20 °C, this sample can be kept for at least 24 months.

6.3 Detection limit

When applying on the gel a sample solution containing 0,067 µg N, about 0,02 g/100 g protein equivalents of ßLG, BSA and α-lactalbumin may be detected ; when applying 1 µg N, about 1 mg/100 g may be detected.

7 ANALYTICAL FLOW SHEET

See Enclosure 2.

8 BIBLIOGRAPHICAL REFERENCES

- S. Lengweiler, M. Braun: Technical Report KR-TR920008: SDS-PAGE with PhastSystem by Pharmacia
- C. Hischenhuber: Internal note (R&D-R/QS/CHi/ap, 16.8.94: Rapid tests for release of LHA)

9 APPROVAL OF METHOD

Approved by	Initials	Date
Author	CH	24.10.
Team Leader	CH	24.10.96
Head of Department		25.10.96
SBU		28.10.96
BT-QM		28.10.96

10 MENTIONED LI

08.082

11 ENCLOSURES

1 Safety instructions
2 Analytical flow sheet

SAFETY INSTRUCTIONS

CAS N°	COMPOUND	HAZARDS	PRECAUTIONS	DISPOSAL
64-19-7	Acetic acid	Corrosive. Flammable.	Use in a chemical fume hood. Avoid contact.	Solvent waste
62625-28-9	Bromophenol blue	Irritant.	Avoid contact.	Chemical waste, no special precautions
27565-41-9	Dithiothreitol (1,4 Dithio-D,L-threitol)	Irritant. May cause headache, nausea and vomiting.	Use in a chemical fume hood. Avoid contact. Avoid inhalation.	Chemical waste, no special precautions for small quantities
61-90-5	Ethanol	Contains methanol: ingestion or contact with eyes may cause blindness. Flammable.	Use with adequate ventilation.	Chemical waste, no special precautions
50-00-0	**Formaldehyde**	Extremely toxic. May be fatal if inhaled. Carcinogen. Mutagen. Irritant. Lacrymator.	Use in a chemical fume hood. Avoid contact. Avoid inhalation. Avoid repeated or prolonged exposure.	Chemical waste, no special precautions for small quantities
111-30-8	Glutaraldehyde	Toxic. Corrosive.	Use in a chemical fume hood. Avoid contact.	Solvent waste
7647-01-0	**Hydrochloric acid**	Highly toxic. Corrosive.	Use concentrated acid only in a chemical fume hood. Avoid contact.	Neutralise.
144-48-9	**Iodoacetamide**	Toxic. Irritant. Sensitiser. Possible teratogen. May cause headache, nausea and vomiting.	Use in a chemical fume hood. Avoid contact. Avoid inhalation. Avoid prolonged exposure.	Chemical waste, no special precautions for small quantities
67-56-1	Methanol	Toxic. Ingestion or contact with eyes may cause blindness. Extremely flammable.	Use with adequate ventilation. Avoid contact.	Solvent waste
7761-88-8	Silver nitrate	Highly toxic.	Use in a chemical fume hood. Avoid contact	Recycle material in elemental state / Chemical waste, no special precautions for small quantities
127-09-3	Sodium acetate	Irritant.	Avoid contact. Avoid inhalation.	Chemical waste, no special precautions
497-19-8	Sodium carbonate	Irritant. May cause headache, nausea and vomiting.	Avoid contact. Avoid inhalation.	Chemical waste, no special precautions for small quantities
151-21-3	Sodium dodecyl sulfate	Irritant. Sensitiser. May cause headache, nausea and vomiting.	Avoid contact. Avoid inhalation.	Chemical waste, no special precautions for small quantities
10102-17-7	Sodium thiosulfate pentahydrate	Irritant.	Avoid contact. Avoid inhalation. Avoid prolonged exposure. Use with adequate ventilation.	Chemical waste, no special precautions
77-86-1	Tris(hydroxymethyl)amino-methane	Irritant.	Avoid contact. Avoid inhalation.	Chemical waste, no special precautions for small quantities

Bold: Particularly toxic - Special precautions required

ANALYTICAL FLOW SHEET

Steps

Critical points

Weigh test portion:

Powders : 61,2 mg if product contains 1,96 % TN
Starches : 14,1 mg if finished product 2,38 % TN and 14 % starch
Liquids : 58,0 g if product contains 0,26 % TN
Fill up to the mark with water

Incubation :

Powders and starches (5.2.1): add 1 ml incubation buffer **4.1.6**
Liquids and hydrolysates (5.2.2): 320 µl sample sol.
+ 80 µl incubation buffer **4.1.5**
Prot.ref.sol.(4.4): content of vial + 1 000 µl incubation buffer **4.1.6**

10 min. at 100 °C or 15 min. at 95 °C

Alkylation :

Prot.ref.sol.(4.4): add 200 µl alkylation sol. **4.2.2**
Powders and starches (5.3): add 200 µl alkylation sol. **4.2.2**
Liquids and hydrolysates (5.3): add 80 µl alkylation sol. **4.2.2**

20 min. at room temperature in the dark

Dilutions:

Protein ref. solutions:
a) 0,8 ng/µl: 50 µl sol. 4.4 + 4,95 ml sol. 4.1.7
b) 0,08 ng/µl: 1 volume sol. a) + 9 volumes sol. 4.1.7

Starches:
a) 0,067 µg N/µl: 50 µl sol. obtained under 5.3 + 700 µl sol. 4.1.7
b) 0,033 µg N/µl: 1 volume sol. a) + 1 volume sol. 4.1.7

Powders and liquids - hypoallergenic formulas, hydrolysates (H.A.):
a) 0,067 µg N/µl: 50 µl sol. obtained under 5.3 + 700 µl sol. 4.1.7
b) 0,033 µg N/µl: 1 volume sol. a) + 1 volume sol. 4.1.7

Powders and liquids - extensively hydrolysed formulas (e.g. Alfaré), hydrolysates (e.g. LAD):
a) 1,0 µg N/µl: solution obtained under 5.3 as is
b) 0,5 µg N/µl: 1 volume sol. a) + 1 volume sol. 4.1.7

Steps	Critical points
Electrophoresis: Refer to Separation program	- Wear non powdered gloves - For PHASTSYSTEM use, refer to the Pharmacia instruction manual - Do not forget to remove the protecting film when gels are ready on the gel bed area - Keep separation compartment closed to prevent contaminations (dust,...) - Steady hand and no air bubbles when applying samples (parafilm) and when loading them into the sample applicator
Silver staining Development: Refer to Development program	- For preparation of solutions, use glass flasks treated with detergent and rinsed with distilled water - Prepare Fixing, Staining and Developing solutions at least 48 hours before use - Before developing, rinse the system with distilled water and with working solutions - Place the gels face to face into the development chamber
Drying: Allow the gels to dry at room temperature	
Evaluation	